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A Dose-Response Relationship Between the Frequency of p53 Mutations and Tobacco Consumption in Lung Cancer Patients

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Mutations of the p53 tumor suppressor gene are frequent in lung cancers. It is suggested that p53 mutations are associated with smoking-induced lung carcinogenesis. We examined p53 mutations in 53 lung cancers by analyzing reverse transcription-polymerase chain reaction-single strand conformation polymorphism (RT-PCR-SSCP) to ascertain the association between p53 mutations and smoking. Twenty-five (47%) of 53 lung cancers carried p53 mutations. A discriminant analysis showed that the Brinkman index (0.156) and gender (0.140) significantly influenced p53 mutations. Furthermore, there was a dose-response relationship between the quantity of cigarettes consumed and the frequency of p53 mutations in lung cancer patients ($P < 0.001$). In patients with adenocarcinoma, the frequency of p53 mutations correlated with the amount of the tobacco smoked ($P < 0.05$). We suggest that the p53 gene is a target of particular carcinogen in tobacco smoke. © 1996 Wiley-Liss, Inc.

KEY WORDS: **lung cancer, p53 gene, RT-PCR-SSCP, smoking**

INTRODUCTION

In Japan, lung cancer will become the leading cause of death due to cancer in the 21st century [1]. Tobacco smoking has emerged as the important risk factor in development of lung cancer. The risk for lung cancer increases with the number of cigarettes smoked and the duration of smoking [2].

The development of molecular biology has helped elucidate the process of carcinogenesis. Some chemical carcinogens form DNA adducts by covalently binding to nucleic acids to produce somatic gene mutations. These mutations are largely responsible for activating proto-oncogenes and inactivating tumor suppressor genes. It is suggested that six or more independent mutational events convert normal cells into malignant cells [3,4].

Mutations of the dominant oncogene, K-*ras*, and the tumor suppressor gene, p53, are frequently found in lung cancers [5–12]. Mutations in both the p53 gene and K-*ras* oncogene in lung cancers are most commonly G to T transversions, which can be caused by cigarette smoking (benzo [a] pyrene exposure) [9,12,13,14]. This evidence suggests that K-*ras* and p53 mutations are associated with smoking-induced lung carcinogenesis.

We examined p53 mutations in 18 lung cancers by means of reverse transcription-polymerase chain reaction-single strand conformation polymorphism (RT-PCR-SSCP) and sequencing, and showed that p53 mutations are associated with lifetime tobacco consumption [10]. Here, we examined p53 mutations in 35 additional lung cancers to clarify 1) whether p53 mutations are associated with cigarette consumption, 2) whether there is an association between p53 mutations and smoking according to the histology of the lung cancer, and 3) whether p53 mutations can be used as an indicator of lung cancer prognosis.

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TABLE I. Clinical Findings in 53 Lung Cancers

Age	63.6 ± 9.6 (38–81)	T factor			
B.I.[a]	827 ± 647 (0–2,400)	T1	15	T3	4
Sex		T2	31	T4	3
Male	37	N factor			
Female	16	N0	24	N2	21
Histology		N1	7	N3	1
Squamous cell carcinoma	19	Disease stage			
Adenocarcinoma	24	I	20	III A	22
Large cell carcinoma	4	II	6	III B	3
Small cell carcinoma	6			IV	2

[a]B.I.: the number of cigarettes per day X years.

MATERIALS AND METHODS

Patients

Tumors and normal lung tissues were obtained from 53 lung cancer patients at surgery at The Second Department of Surgery, School of Medicine, The University of Tokushima, Japan, from March 1986 to November 1990. The patients' clinical information (age, Brinkman index [B.I. = the number of cigarettes per day × years], gender, histology, T, N factor, and stage) is summarized in Table I. We used the Union International Contre le Cancer (UICC) TNM staging system [15]. The tumor histology was determined according to the 1981 World Health Organization (WHO) classification of lung tumors [16]. To clarify whether or not the frequency of p53 mutations is correlated with the amount of tobacco smoked, we divided the patients into four groups; nonsmokers, smokers with a B.I. of 600 or less, smokers with a B.I. of 600–1,000, and smokers with a B.I. over 1,000.

RT-PCR-SSCP

Total RNA was extracted from frozen samples by a single-step procedure using an acid guanidinium thiocyanate-phenol-chloroform mixture [17]. The first strand of cDNA was synthesized with a p53-specific oligonucleotide primer as described [10]. Point mutations in the p53 gene in codon 101 through 300 (midway in the 4th exon to midway in the 8th exon) were detected by PCR-SSCP (most mutations fall within this region of the p53 gene) [18,19]. Two fragments (codons 101–200 and 201–300) were amplified with the following primer pairs:

5′-CATCTTCTGTCCCTTCCCAG-3′ and
5′-TCCAAATACTCCACACGCAA-3′ for codons 101–200
5′-TTATCCGAGTGGAAGGAAAT-3′ and
5′-CTCGCTTAGTGCTCCCTGG-3′ for codons 201–300.

These primer pairs were labeled with [γ-^{32}P] dATP and PCR was performed as described [20]. The PCR products were diluted with loading buffer, heated, denatured, and loaded onto a 6% polyacrylamide gel with or without 5% glycerol. Electrophoresis proceeded at room temperature and at a constant power of 40 W. Thereafter, the gel was transferred to Whatman 3MM paper and dried. Autoradiography with Kodak X-Omat AR film was performed at room temperature for 1 hour. The sensitivity of PCR-SSCP for detecting point mutations is more than 89% for 300 to 400 bp fragments, and the specificity is 100% [21]. In this study, when alterations of the p53 gene were well detected by SSCP, they were not confirmed by sequencing.

Statistical Analysis

The association between the incidence of p53 mutations and several clinical factors was compared by means of the chi-square test or *t* test (unpaired). Furthermore, discriminant analysis by the SAS statistical package identified clinical factors that independently or together significantly influenced p53 mutations. Correlations between the frequency of p53 mutations and the tobacco consumption were examined by means of the Cochran-Armitage test. The Kaplan-Meier method was used to estimate survival potential as a function of time, and survival differences were analyzed by the log rank test.

RESULTS

Detection of Point Mutations of the p53 Gene by RT-PCR-SSCP

We detected p53 mutations in tumor samples from 25 (47%) of 53 lung cancer patients by RT-PCR-SSCP. Representatives of RT-PCR-SSCP (DNA fragments carrying codons 101–200) are shown in Figure 1. The bands from two lung cancer patients (24, 33) showed slightly different mobilities from those of the normal lung. The mobility of the bands in sample 29 was quite different.

p53 Mutations and Cigarette Smoking (All Samples)

Statistically significant relationships were found between p53 mutations and the B.I. ($P = 0.003$). The discriminant analysis showed that the B.I. factor (0.156) greatly influenced p53 mutations among five clinical factors (Table II). Furthermore, the frequency of p53 mutations correlated with the amount of the tobacco smoked

TABLE II. Abnormality of p53 Gene and Clinical Findings†

Characteristics	p53 mutation			
	Positive	Negative		R^{2a}
Age (mean ± SD)	66.6 ± 5.5	61.0 ± 11.6	$P = 0.035$*	0.084
B.I. (mean ± SD)	1,095 ± 515	588 ± 668	$P = 0.003$*	0.156
Sex				
Male	22 (60%)	15	$P = 0.015$**	0.140
Female	3 (19%)	13		
Histology				
Squamous cell carcinoma	11 (58%)	8	$P = 0.012$**	0.009
Adenocarcinoma	6 (25%)	18		
Large cell carcinoma	4 (100%)	0		
Small cell carcinoma	4 (67%)	2		
Disease stage				
I, II	11, 4 (55, 67%)	9, 2	P = N.S.**	0.007
IIIA, IIIB	7, 1 (32, 33%)	15, 2		
IV	2 (100%)	0		

†N.S. = not significant.
[a]Coefficient of multiple determination (discriminant analysis).
*t test (unpaired).
**Chi-square test.

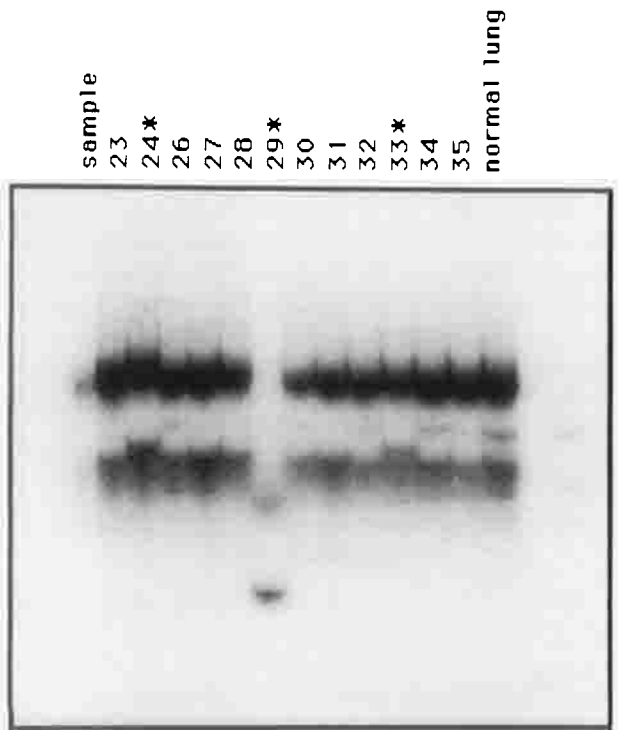

Fig. 1. Electrophoresis of DNA fragments carrying codons 101–200 of the p53 gene by SSCP. The bands in samples from three lung cancer patients (24, 29, 33) show different mobilities from those of normal lung.

($P < 0.001$; Cochran-Armitage test). The more cigarettes that were consumed, the more frequent were the p53 mutations among lung cancer patients (Fig. 2). The p53 mutations were identified in 1 (7.7%) of the 13 lung cancers obtained from nonsmokers, in 3 (42.9%) of the 7 lung cancers from smokers with a B.I. of 600 or less, in 9 (60%) of the 15 lung cancers from smokers with a B.I. of over 600 but less than 1,000, and in 12 (66.7%) of the 18 lung cancers from smokers with a B.I. over 1,000.

p53 Mutations and Cigarette Smoking (Squamous Cell Carcinoma vs. Adenocarcinoma)

There is a relationship between the frequency of p53 mutations and the amount of the tobacco smoked according to histology of the lung cancer (Fig. 3). In patients with squamous cell carcinoma, the frequency of p53 mutations of smokers was constant (about 60%) independently of the amount of smoking. These mutations were identified in two (67%) of the three lung cancers from smokers with a B.I. of 600 or less, in five (63%) of the eight from smokers with a B.I. of over 600 but less than 1,000, and in four (57%) of the seven from smokers with a B.I. over 1,000. However, in patients with adenocarcinoma, the frequency of p53 mutations in nonsmokers was significantly less than that in smokers. The frequency of p53 mutations in the group of nonsmokers was 8% (1 of 12), while that in the group of smokers was 50% (5 of 10; $P < 0.05$). The frequency of p53 mutations correlated with the amount of the tobacco smoked ($P < 0.05$; Cochran-Armitage test). The p53 mutations were identified in none (0%) of the two lung cancers from smokers with a B.I. of 600 or less, in one (33%) of the three from smokers with a B.I. of over 600 but less than 1,000, and in four (57%) of the seven from smokers with a B.I. over 1,000. The smokers with a B.I. over 1,000 and adenocarcinoma

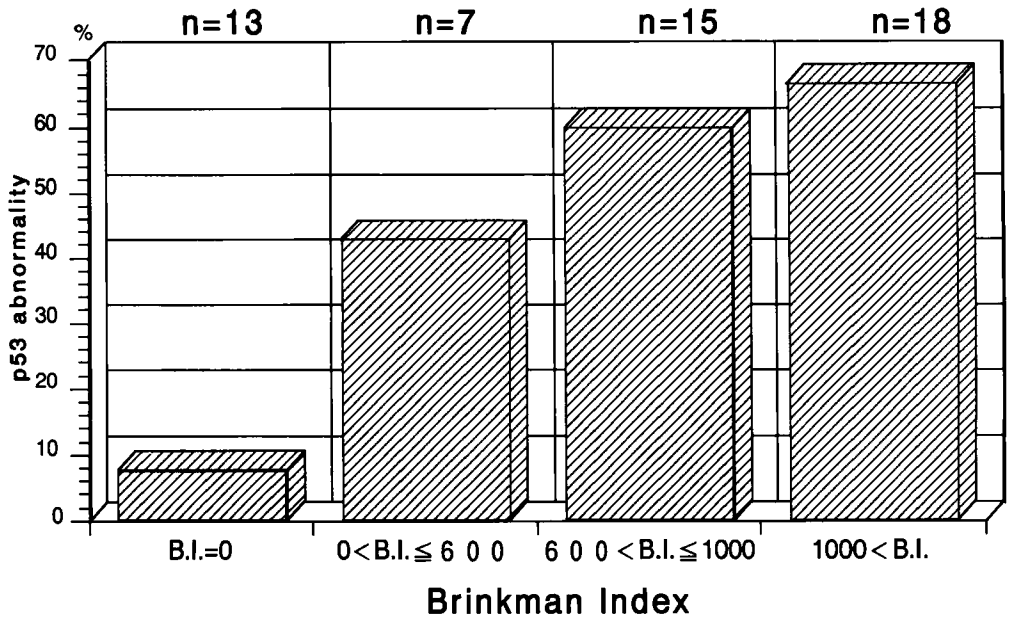

Fig. 2. Mutation of the p53 gene and the B.I. (all samples). $P < 0.001$; Cochran-Armitage test.

had the same frequency of p53 mutations as the smokers with squamous cell carcinoma.

p53 Mutations and Other Clinical Findings

Statistically significant relationships were found between p53 mutations and age, gender, or histology (Table II). However, there was no relationship between p53 mutation and disease stage, T factor, or N factor. It was shown that gender (0.140) influenced p53 mutations as well as the B.I. (0.156) according to discriminant analysis.

Prognostic Significance of p53 Mutations in Non-Small Cell Lung Cancers (NSCLC)

The Kaplan-Meier survival curve in stage I-IIIA NSCLC patients demonstrated a tendency toward a poor prognosis in patients with p53 mutations than in those without p53, but the difference was not significant ($P = .088$ by log rank test, Fig. 4A). The association was significant between the presence of p53 mutations and shortened survival among patients with advanced-stage (IIIA) NSCLC ($P = 0.041$ by log rank test, Fig. 4B). However, there was no significant association at the early-stage (I or II) NSCLC ($P = 0.495$ by log rank test, Fig. 4C).

DISCUSSION

We demonstrated that tobacco consumption is associated with mutational changes in the p53 gene in lung cancers. Furthermore, this study revealed a dose-response relationship between the quantity of tobacco consumed and the frequency of p53 mutations in lung cancer patients ($P < 0.001$). This dose-response relationship supports the notion that some chemical carcinogens produce somatic gene mutations and inactivate tumor suppressor genes, resulting in carcinogenesis [4]. It is possible that the p53 gene is a target of a particular carcinogen in tobacco smoke, and that it is one of the carcinogenesis-associated genes in smoking-induced lung cancers. Suzuki et al. [12] have also reported that p53 mutations are closely associated with lifetime cigarette consumption in Japan. Gosney et al. [22] have reported that overexpression of p53 protein is associated with cigarette consumption in England. However in America, Chiba et al. [9] reported that there was no significant association between smoking and p53 mutations in NSCLC. Analysis of a large number of p53 mutations in lung cancers with or without smoking might explain this discrepancy. One explanation may be a hereditary variation in the manner in which carcinogens are activated or inactivated in each population [4,23].

This study revealed that p53 mutational changes are quite frequent in smokers with adenocarcinoma. Furthermore, the dose-response relationship between the quantity of tobacco consumed and the frequency of p53 mutations in lung cancer patients is not found in those with squamous cell carcinoma, but with adenocarcinoma. The frequency of p53 mutations in smokers was significantly higher than that in nonsmokers with adenocarcinoma ($P < 0.05$). Westra et al. [24] have revealed that the frequency of p53 overexpression in both current smokers and ex-smokers was higher than that in nonsmokers (23 of 58 [40%] vs. none of 12 [0%]). The same tendency is said for the K-*ras* point mutations in lung adenocarci-

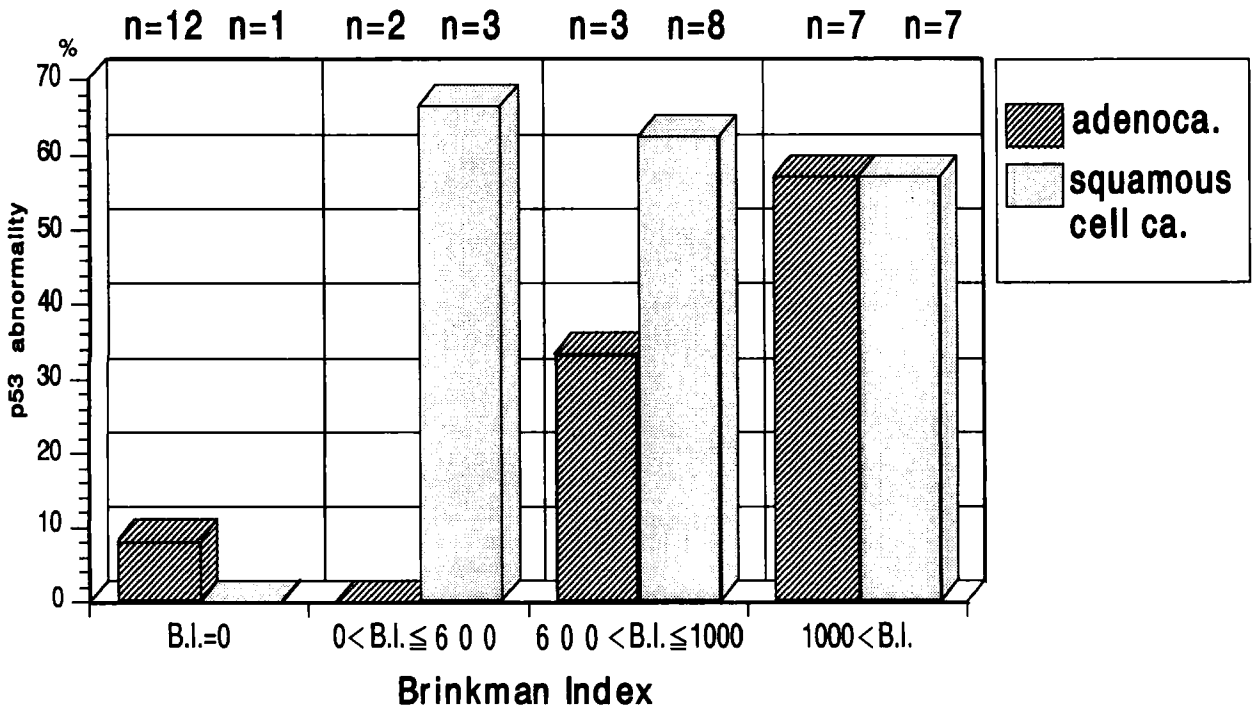

Fig. 3. Mutation of the p53 gene and the B.I. (squamous cell carcinoma vs. adenocarcinoma). Cochran-Armitage test; adenocarcinoma, $P < 0.05$; squamous cell carcinoma, not significant.

noma. Slebos et al. [13] have reported that the frequency of K-*ras* mutations in smokers (30%) is significantly higher than that in nonsmokers (7%). These studies suggest that some carcinogens of tobacco smoke activate the K-*ras* oncogene or inactivate the p53 tumor suppressor gene in the lung, and that it is one of the risk factors for developing lung adenocarcinoma. In general, lung adenocarcinoma is weakly associated with tobacco smoking [25]. We think that some carcinogens other than tobacco smoke influence the carcinogenesis of lung adenocarcinoma so much that the effects of smoke may be masked.

This study demonstrated that independently of the quantity of smoking consumption, the frequency of p53 mutations was constant (about 60%) in smokers with squamous cell carcinoma. This result suggested that the frequency of p53 mutations may become maximal after a certain amount of exposure to tobacco smoke. In smokers with adenocarcinoma, the incidence of p53 mutations increased according to the increase in tobacco consumption ($P < 0.05$). Particularly, the frequency of p53 mutations in smokers with a B.I. over 1,000 with adenocarcinoma reached the same frequency (about 60%) as that of smokers with squamous cell carcinoma. These differences between adenocarcinoma and squamous cell carcinoma may be due to where the two subtypes of lung cancer arise. About 75–95% of squamous cell carcinoma arises in the basal cells of the subsegmental or larger bronchial epithelium which is easily exposed to carcinogen particles. On the other hand, adenocarcinoma arises in the periphery of the lung which is not so likely to be exposed.

Statistical analysis showed that there was no significant correlation between the p53 mutations and T, N factor or disease stage. Sozzi et al. [26] have detected p53 mutations in severe dysplasia of the bronchial mucosa adjacent to lung cancer. These data provide evidence that p53 mutations do not occur during a progressive process, but at the early stage of lung carcinogenesis. The discriminant analysis revealed that the B.I. and gender among clinical factors correlated to the highest degree with p53 mutations in lung cancers. Thirteen of 16 females were nonsmokers, and none of 37 males was a nonsmoker in this study. Gender was superimposed on smoking factor.

This study revealed that p53 mutations in advanced stage NSCLC were associated with shorter survival, but those in the early stage were not. This finding was in agreement with that of Mitsudomi et al. [27]. However, in stage I or II NSCLC, the findings of our study and that of Mitsudomi et al., that p53 mutations did not correlate with survival, were incompatible with those of Horio et al. [28]. In America, Quinlan et al. [29] have reported that p53 accumulation in lung cancers at stage I and II had a statistically significant negative prognostic value. Survival analysis of more patients with stage I or II NSCLC may clarify this discrepancy.

In conclusion, we found a dose-response relationship between the quantity of tobacco consumed and the frequency of p53 mutations among lung cancer patients, and

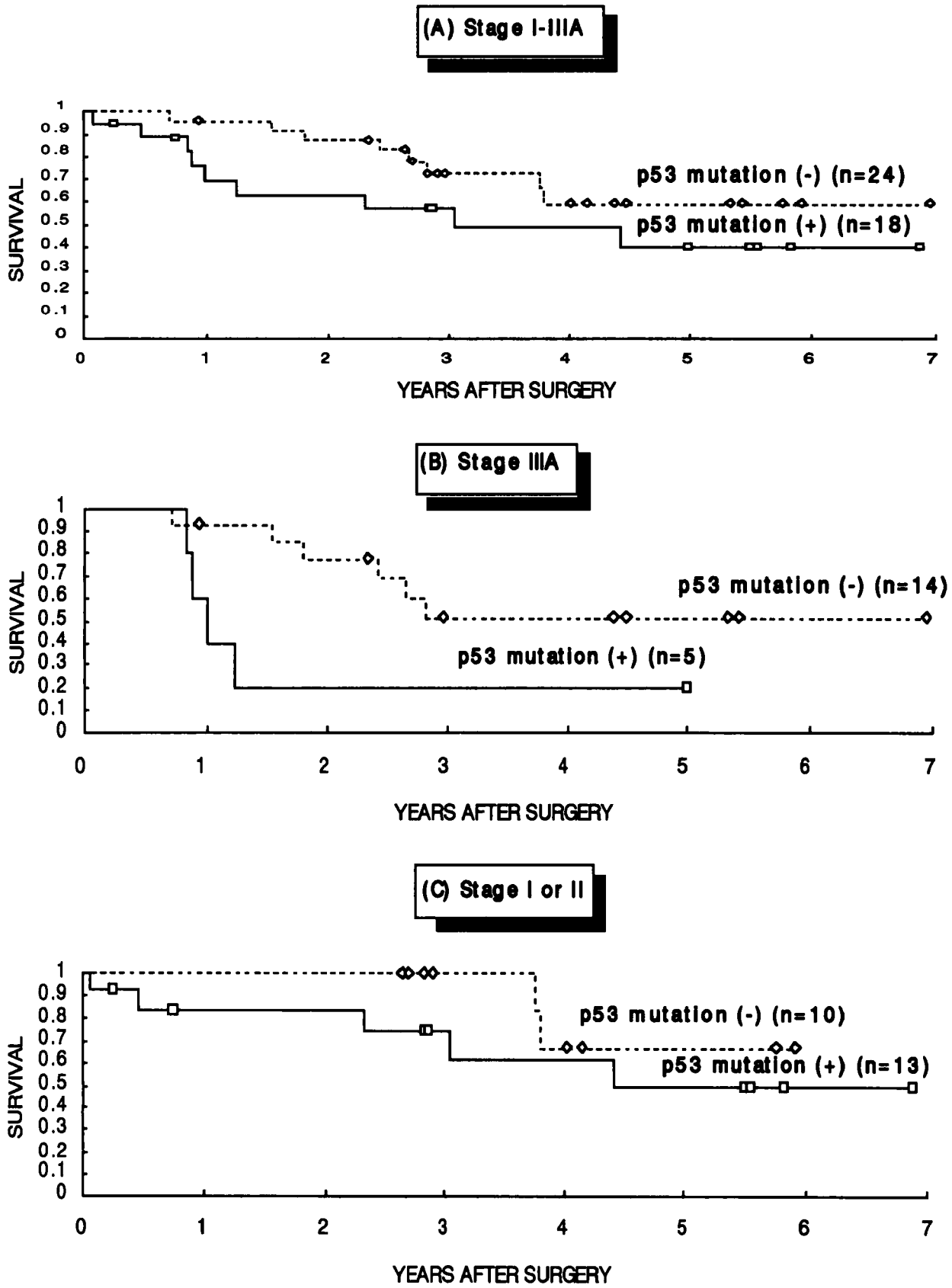

Fig. 4. Survival curves of NSCLC with or without p53 mutation. **A:** Stage I-IIIA ($P = 0.088$ by log rank test). **B:** Stage IIIA ($P = 0.041$ by log rank test). **C:** Stage I or II ($P = 0.495$ by log rank test).

this relationship is not found in patients with squamous cell carcinoma, but in those with adenocarcinoma. We suggest that the p53 gene is a target of a particular carcinogen in cigarette smoke and that it is one of the carcinogenesis-associated genes in smoking-induced lung cancers.

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